

VU Research Portal

Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity

Legler, J.; Zeinstra, L.M.; Schuitemaker, F.; Lanser, P.H.; Bogerd, J.; Brouwer, A.; Vethaak, A.D.; de Voogt, P.; Murk, A.J.; van der Burg, B.

published in

Environmental Science and Technology
2002

DOI (link to publisher)

[10.1021/es010323a](https://doi.org/10.1021/es010323a)

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

Legler, J., Zeinstra, L. M., Schuitemaker, F., Lanser, P. H., Bogerd, J., Brouwer, A., Vethaak, A. D., de Voogt, P., Murk, A. J., & van der Burg, B. (2002). Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. *Environmental Science and Technology*, 36, 4410-4415.
<https://doi.org/10.1021/es010323a>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:

vuresearchportal.ub@vu.nl

Comparison of in Vivo and in Vitro Reporter Gene Assays for Short-Term Screening of Estrogenic Activity

JULIETTE LEGLER,^{†,‡,§}
 LAURA M. ZEINSTRÄ,[†]
 FEMKE SCHUITEMAKER,[†]
 PETER H. LANSER,[†] JAN BOGERD,[⊥]
 ABRAHAM BROUWER,^{‡,§}
 A. DICK VETHAAK,[#] PIM DE VOOGT,^{||}
 ALBERTINKA J. MURK,[‡] AND
 BART VAN DER BURG^{*.†}

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, Division of Toxicology, Wageningen University, P.O. Box 8000, 6700 EA Wageningen, The Netherlands, Department of Experimental Zoology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands, Ministry of Transport, Public Works and Water Management, National Institute for Coastal and Marine Management, P.O. Box 8039, 4330 EA Middelburg, The Netherlands, and Department of Environmental Chemistry and Toxicology, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Functional in vitro and in vivo reporter gene assays have recently been developed for the rapid determination of exposure to (xeno)estrogens. The in vitro estrogen receptor (ER)-mediated chemically activated luciferase gene expression (ER-CALUX) assay uses T47D human breast cancer cells stably transfected with an ER-mediated luciferase gene construct. In the in vivo assay, transgenic zebrafish are used in which the same luciferase construct has been stably introduced. In both assays, luciferase reporter gene activity can be easily quantified following short-term exposure to chemicals activating endogenous estrogen receptors. The objective of this study was to compare responses by known (xeno)estrogenic compounds in both assays. Exposure to the (xeno)estrogens estradiol (E2), estrone, ethynylestradiol (EE2), *o,p'*-DDT, nonylphenol (NP), and di(2-ethylhexyl)phthalate (DEHP) revealed that EE2 was the most potent (xeno)estrogen tested and was 100 times more potent than E2 in the transgenic zebrafish assay, whereas in the in vitro ER-CALUX assay, EE2 and E2 were equipotent. Although the xenoestrogens *o,p'*-DDT and NP were full estrogen agonists in the in vitro ER-CALUX assay, only *o,p'*-DDT demonstrated weak dose-related

estrogenic activity in vivo. To determine if differences in reporter gene activity may be explained by differential affinity of (xeno)estrogens to human and zebrafish ERs, full-length sequences of the zebrafish ER subtypes α , β , and γ were cloned, and transactivation by (xeno)estrogens was compared to human ER α and ER β . Using transiently transfected recombinant ER and reporter gene constructs, EE2 also showed relatively potent activation of zebrafish ER α and ER β compared to human ER α and ER β . Zebrafish ER β and ER γ showed higher transactivation by (xeno)-estrogens relative to E2 than human ER β .

Introduction

The presence of hermaphrodite fish has been increasingly reported in wild fish populations in rivers, estuaries, and coastal waters (1–4). These effects have been associated with exposure of fish in the aquatic environment to natural and xenobiotic compounds with the same mode of action as the estrogen steroid hormones [the so-called “(xeno)estrogens”]. The focus on (xeno)estrogens in the aquatic environment can be largely attributed to the finding that effluents from wastewater treatment plants (WTP) contain (xeno)estrogenic chemicals, such as the natural estrogens 17 β -estradiol (E2) and estrone (E1), the synthetic estrogen 17 α -ethynylestradiol (EE2), and xenoestrogens such as the alkylphenol surfactants at levels sufficient to invoke estrogenic effects in fish (reviewed in ref 5). However, routine chemical analysis of (xeno)-estrogens in complex mixtures such as WTP effluent is difficult and hampered by high costs. Only known compounds are included in the analysis, and no account is taken of the biological effects of mixtures of chemicals. The use of bioassays to rapidly screen the exposure and demonstrate potential effects of (xeno)estrogens can provide an ideal means to determine the total estrogenic potency of mixtures of substances. Recently, extensive efforts to develop screening assays to detect exposure of (xeno)estrogenic compounds on wildlife have been undertaken (reviewed in refs 6 and 7). In our laboratories, two new assays for estrogenic activity have been recently developed: the in vitro estrogen receptor-mediated chemically activated luciferase gene expression (ER-CALUX) assay using human T47D breast cancer cells (8) and the in vivo transgenic zebrafish assay (9). In both assays, an ER-mediated luciferase reporter gene construct containing three estrogen response elements (ERE) has been stably introduced and integrated in the genome of the T47D cells and transgenic zebrafish. In the ER-CALUX assay, exposure of cells to (xeno)estrogens results in uptake of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and, consequently, binding of the ligand–receptor complex to EREs present in the promoter region of the luciferase gene. Luciferase protein is then induced and is easily measured by lysing the cells, adding luciferin substrate, and measuring light photon production. In the transgenic zebrafish assay, luciferase is induced according to the same principle, but exposure to test substances takes place via the water phase. Therefore, the environmental chemistry, bioavailability, and toxicokinetics of the test substance in vivo determine the ultimate exposure of target cells in the transgenic fish. Luciferase protein will be induced only in target cells in which the test substance is bioavailable and active endogenous ERs and cofactors necessary for ER transcription are present. Therefore, the response of target tissues and specific life stages to (xeno)-

* Corresponding author phone: + 31 30 2121923; fax: + 31 30 2516464; e-mail: bvdv@niob.knaw.nl.

[†] Netherlands Institute for Developmental Biology.

[‡] Wageningen University.

[§] Present address: Institute for Environmental Studies, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

[⊥] Utrecht University.

[#] Ministry of Transport, Public Works and Water Management.

^{||} University of Amsterdam.

estrogen exposure can be determined in the transgenic zebrafish.

The objective of this study was to compare and contrast the *in vitro* ER-CALUX and *in vivo* transgenic zebrafish assays as short-term screens for environmentally relevant (xeno)-estrogens. As differential binding and activation of compounds by human and zebrafish estrogen receptors may explain differences in responses between the two assays, transient transfection experiments were carried out with ER α and ER β subtypes cloned from both species, as well as the novel zebrafish ER γ subtype. The use of *in vitro* and *in vivo* reporter gene assays as screens for estrogenic activity is discussed.

Experimental Section

Test Chemicals. 17 β -Estradiol (E2, 99%) and ethanol (100%, p.a.) were purchased from Sigma Chemical Co. Estrone (E1, p.a.) was from Brunschwig. Ethynylestradiol (EE2, 98%) was purchased from Aldrich. 4-Nonylphenol (NP, 91%) and di-(2-ethylhexyl)phthalate (DEHP, 98% purity) were from TCI, Japan. The organochlorine insecticide *o,p'*-dichlorodiphenyl trichloroethane (*o,p'*-DDT) was kindly provided by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). Dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade) was purchased from Acros.

ER-CALUX Assay. The development of the stably transfected T47D cells and the ER-CALUX assay procedure is described in detail elsewhere (8). Briefly, T47D.Luc cells stably transfected with the DNA construct pERetata-Luc containing a luciferase reporter gene regulated by three tandem repeats of the consensus ERE oligonucleotide (GAGCTTAGGTCACTGTGACCT) upstream of the minimal human E β TATA promoter sequence (GGGTATATAAT) were plated in clear plastic 96 well plates (Nucleon). Cells were plated at a density of 5000 cells per well in 0.1 mL of assay medium containing DMEM-F12 without phenol red and 5% dextran-coated charcoal-stripped fetal calf serum (DCC-FBS). Following 24 h of incubation, medium was renewed and the cells were incubated for another 24 h. The medium was then removed, and the cells were dosed in triplicate by addition of the dosing medium containing the chemical dissolved in DMSO (maximum 0.2%). A complete E2 dose-response curve was included with each assay. Control wells, solvent control wells, and E2 calibration points (6 and 30 pM) were included in triplicate on each plate. Following 24 h of treatment, cells were lysed in 50 μ L of Triton lysis buffer, pH 7.8 (containing 1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO $_4$, 4 mM EGTA, and 1 mM DTT) for a minimum of 1 h with gentle shaking at 4 $^{\circ}$ C. A sample of 25 μ L of lysate was then transferred to a black 96 well plate (Costar), and 25 μ L of luciferin solution (Lucite, Packard) was added per well. Luciferase activity was assayed in a scintillation counter (Top Count, Packard) for 0.1 min per well.

Transgenic Zebrafish Assay. The recent development of the transgenic zebrafish stably expressing pERetata-Luc is described elsewhere (9). For the assays, heterozygous transgenic juveniles of the F4 generation of the age of 4–5 weeks were used. Juvenile transgenic zebrafish undergoing gonad differentiation were used because this period was previously shown to be the most responsive to estrogens during development (9). Juvenile fish ($n = 5$ –6) were exposed for 96 h in 200 mL of acclimated tap water (26–27 $^{\circ}$ C) in all-glass aquaria. The chemical to be tested was added to the water at a volume not exceeding 0.01% solvent. Fish were fed once daily with live brine shrimp (*Artemia salinas*). Test medium was renewed for 50% daily. After termination of the exposure, fish were sacrificed, transferred to Eppendorf vials, and immediately frozen at –80 $^{\circ}$ C. To assay luciferase, Eppendorf vials containing fish were transferred to ice, 500 μ L of cold Triton lysis buffer was added, and the fish were homogenized

using a micropestle (Eppendorf). Following centrifugation at 12000 rpm for 15 min at 4 $^{\circ}$ C, duplicate samples of 25 μ L of supernatant were assayed in a luminometer (LUMAC) with automatic injection of 100 μ L of luciferin substrate, pH 7.8 [containing 20 mM tricine, 1 mM (MgCO $_3$)Mg(OH) $_2$ ·5H $_2$ O, 2.67 mM MgSO $_4$, 0.1 mM EDTA, 33.7 mM DTT, 270 μ M coenzyme A, 470 μ M luciferin, and 530 μ M ATP]. Light units measured by the luminometer were normalized for protein content, which was measured according to the method of Bradford (10).

Human and Zebrafish ER Transactivation. The cloning of full-length zebrafish (zf) ER α , zfER β , and zfER γ is described elsewhere (Zeinstra et al., in preparation). The DNA sequences of these receptors have been submitted to GenBank. The zf ER constructs pSG5-zfER α , pSG5-zfER β , and pSG5-zfER γ were constructed by insertion of the full-length zfER α , zfER β , and zfER δ cDNAs into the *Eco*RI site of the multiple cloning site of the pSG5 expression vector (Stratagene). Human ER α expression plasmid pSG5-hER α HEGO was kindly provided by Dr. P. Chambon, IGBMC, Strasbourg, France. Human ER β expression plasmid pSG5-hER β was a kind gift from Dr. J. A. Gustafsson, Karolinska Institute, Huddinge, Sweden. Transactivation of these receptor constructs by (xeno)estrogens was tested in the human embryonic kidney (293HEK) cell line, which was acquired from the American Type Culture Collection (ATCC, Rockville, MD), essentially as described elsewhere (11). Briefly, the 293HEK cells were plated at a density of 35000 cells/cm 2 in 24 well plates (Costar). After 30 h, cells were transiently transfected by calcium phosphate precipitation using 0.8 μ g of pERetata-Luc (luciferase reporter construct), 0.4 μ g of pSV $_2$ LacZ (β -galactosidase expression vector), 0.6 μ g of Bluescript SK $^-$ (Stratagene), and 0.2 μ g of receptor construct for a total of 2.0 μ g of DNA per well. Medium was removed 16 h after transfection, and the cells were dosed in triplicate by the addition of assay medium containing the chemical to be tested dissolved in ethanol (maximum 0.1%). Following 24 h of incubation, medium was removed and cells were lysed in 150 μ L of Triton lysis buffer. Luciferase activity was assayed in a 50 μ L sample with the addition of 50 μ L luciferin as described for the ER-CALUX assay above. The β -galactosidase activity was measured to correct for variation in transfection efficiency.

Analysis of Actual Exposure Concentrations. Test chemicals were measured in exposure water in transgenic zebrafish assays at the start ($t = 0$) of an exposure experiment, prior to introducing the transgenic zebrafish, as well as at the end ($t = 96$ h) of the experiment. Test medium samples were ~100 mL. Although tissue hormone (E2, E1, and EE2) concentrations could not be analyzed due to analytical limitations, NP and *o,p'*-DDT levels were analyzed in whole fish homogenates ($n = 5$, total weight ~100 mg) at the start and termination of the experiment.

E2, E1, and EE2 analysis in water was carried out as described in Belfroid et al. (12), with the exception that the quantification of the chromatograms was done with the internal deuterated standard of *d*4-17 β -estradiol. For NP analysis in test medium, water was extracted according to the method described by De Voogt et al. (13). Analysis of NP in zebrafish following 96 h of exposure was done according to the method of Zhao et al. (14). Extracts were analyzed by reversed-phase HPLC with fluorescence detection. For *o,p'*-DDT analysis, exposure water samples were extracted three times with *n*-hexane. As these samples were not filtered prior to extraction, it is possible that *o,p'*-DDT bound to suspended matter (e.g., detritus) in the water column was also extracted, leading to concentrations apparently exceeding nominal concentrations. The extracts were dried over anhydrous Na $_2$ SO $_3$ and analyzed by GC-ECD. Zebrafish were mixed with anhydrous Na $_2$ SO $_3$ and then extracted three times with acetone/*n*-hexane (1:3 v/v). The extract was then filtered

and analyzed with GC-ECD. The extraction efficiency of *o,p'*-DDT was 90–100%.

Data Analysis. Data shown are representative of a minimum of three independent experiments. In the figures, values represent averages of three test wells for in vitro experiments and five to six fish per concentration for the transgenic zebrafish assay. The EC₅₀ values for the ER-CALUX, transient transfections with human and zebrafish ER constructs, and transgenic zebrafish assays were calculated from sigmoid dose–response curves using the curve-fitter of SlideWrite for Windows, version 4.0 (cumulative fit). The *R* of the fit of the curves was >0.99 for the in vitro assays and >0.9 for the transgenic zebrafish assay. EC₅₀ values were calculated by determining the concentration at which 50% of the maximum luciferase activity was reached. Estradiol equivalent factors (EEF) were determined as the ratio EC₅₀(E2)/EC₅₀(test compd). EEQ and EEF values were calculated on the basis of an average of three to five independent experiments.

Results and Discussion

ER-CALUX and Transgenic Zebrafish Assay Characteristics.

The in vitro ER-CALUX assay using stably transfected T47D cells can detect estradiol (E2) at concentrations as low as 0.5 pM and demonstrates an EC₅₀ of 6 pM, with a coefficient of variation of ~5%. Maximal induction (~100-fold relative to solvent controls) is reached at 30 pM E2. The in vivo transgenic zebrafish assay using 96-h-exposed juvenile transgenics has a detection limit of ~300 pM E2 and an EC₅₀ of 10 nM and exhibits higher variation (CV = 20–30%). Maximal fold induction of 1000–3000-fold relative to solvent controls is reached at 100 nM E2. The high biological variation is a reflection of individual fish differences in uptake and metabolism of substances, as well as differences in (gonad) developmental stage and sex. As the phenotypic sex of the transgenic zebrafish is not apparent at the age of fish used, both males and females are assayed together, thereby contributing to the variation in response. Previous studies have reported that male and female transgenics respond differently to E2 exposure (9). Interestingly, a decrease of luciferase activity by high concentrations of (xeno)estrogens can be seen in both assays, particularly in the transgenic zebrafish assay (Figure 1). Down-regulation of ERs or other mechanisms of negative feedback or sublethal toxicity to high doses may cause this decrease.

Relative Responses of the Screening Assays to (Xeno)-estrogens. The ER-CALUX and transgenic zebrafish reporter gene assays demonstrated a dose–response-related increase in luciferase protein expression following exposure to estradiol (E2), estrone (E1), and ethynylestradiol (EE2) (Figure 1). However, the sensitivities of the responses of the two assays differ, and the relative potencies of (xeno)estrogens vary according to the assay used (Table 1). In the ER-CALUX assay, the EC₅₀ values of E2 and E1 were 2–3 orders of magnitude lower than in the transgenic zebrafish (Table 1). E1 induced luciferase in the transgenic zebrafish at similar concentrations as E2 (EEF = 1; Table 1). In the ER-CALUX assay, however, E1 was considerably less potent than E2, with an EEF of 0.2 (Table 1). The apparent higher in vivo potency of E1 relative to E2 in the fish as compared to the in vitro assay has been reported previously. In a recent study, Panter and colleagues (15) demonstrated that E1 induced vitellogenin at concentrations similar to E2 in male fathead minnows. Routledge and colleagues (16) also showed that E1 was only slightly less potent than E2 in inducing vitellogenin in rainbow trout. In both assays, EE2 was the most potent (xeno)estrogen tested with only a 20-fold difference in EC₅₀ between the ER-CALUX (0.005 nM) and transgenic zebrafish (0.1 nM) assays (Table 1; Figure 1). Interestingly, in the transgenic fish, EE2 was 100 times more potent than E2, whereas in the ER-CALUX, EE2 was slightly

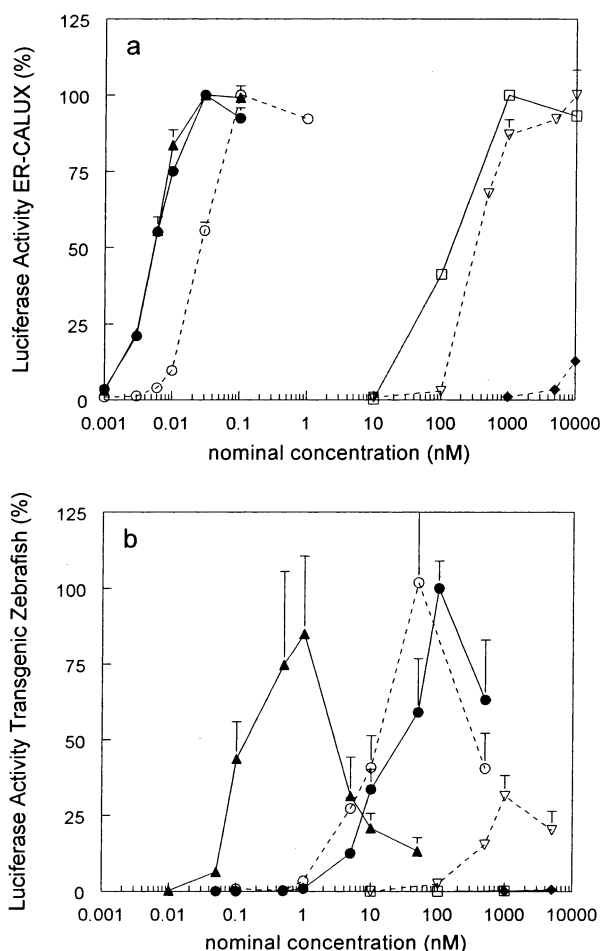


FIGURE 1. Estrogenic potency of (xeno)estrogens estradiol (E2, ●), estrone (E1, ○), ethynylestradiol (EE2, ▲), *o,p'*-DDT (▽), 4-nonylphenol (NP, □), and DEHP (◆) in (A) in vitro ER-CALUX and (B) in vivo transgenic zebrafish reporter gene assays. Data are representative of three independent assays. Values given are average percentage luciferase activity relative to maximal response levels induced by E2; error bars represent standard error of the mean (ER-CALUX, *n* = 3; transgenic zebrafish, *n* = 5–6).

TABLE 1. Estrogenic Potency of (Xeno)estrogens in In Vitro ER-CALUX and in Vivo Transgenic Zebrafish Assays

compd	ER-CALUX		transgenic zebrafish	
	EC ₅₀ ^a (nM)	EEF ^b	EC ₅₀ ^a (nM)	EEF ^b
E2	0.006	1	10	1
EE2	0.005	1.2	0.1	100
E1	0.06	0.1	10	1
<i>o,p'</i> -DDT	390	1.5 × 10 ⁻⁵	450	0.02
NP	260	2.3 × 10 ⁻⁵	>1000 ^c	<0.01
DEHP	>30000 ^c	<2.0 × 10 ⁻⁷	>5000 ^c	<0.05

^a EC₅₀ is nominal concentration at which 50% of maximal response is reached. ^b EEF is the ratio EC₅₀(E2)/EC₅₀(compd). ^c EC₅₀ could not be calculated as no or minimal luciferase induction was observed; higher concentrations caused (cyto)toxicity.

(1.2 times) more potent (Table 2). The high estrogenic activity of EE2 to zebrafish has also been demonstrated in other fish species, such as the medaka (17) and fathead minnow (18), providing even more evidence that the presence of this compound in aquatic environments is of particular concern.

Another dramatic difference in response between the ER-CALUX and transgenic zebrafish assay was found with exposure to xenoestrogenic chemicals. The organochlorine pesticide *o,p'*-DDT induced luciferase activity in a full dose-

TABLE 2. Estrogenic Potency of (Xeno)estrogens To Transactivate (A) Human ER α and ER β and (B) Zebrafish ER α , ER β , and ER γ ^a

compd	A, Human ER			
	human ER α		human ER β	
	EC ₅₀ ^b (nM)	EEF ^c	EC ₅₀ ^b (nM)	EEF ^c
E2	0.009	1	0.08	1
EE2	0.008	1.1	0.14	0.6
E1	0.08	0.1	0.65	0.1
<i>o,p'</i> -DDT	230	3.9×10^{-5}	700	1.1×10^{-4}
NP	110	8.2×10^{-5}	145	5.5×10^{-4}
DEHP	>30000 ^c	$<3.0 \times 10^{-7}$	>30000 ^c	$<3.0 \times 10^{-7}$

compd	B. Zebrafish ER					
	zebrafish ER α		zebrafish ER β		zebrafish ER γ	
	EC ₅₀ ^b (nM)	EEF ^c	EC ₅₀ ^b (nM)	EEF ^c	EC ₅₀ ^b (nM)	EEF ^c
E2	0.38	1.0	0.50	1.0	0.10	1.0
EE2	0.10	3.8	0.10	5.0	0.04	2.5
E1	1.01	0.4	4.08	0.1	1.14	0.1
<i>o,p'</i> -DDT	1800	2.1×10^{-4}	5010	9.9×10^{-5}	2050	4.9×10^{-5}
NP	420	9.0×10^{-4}	1480	3.4×10^{-4}	670	1.5×10^{-4}
DEHP	>30000 ^d	$<1.3 \times 10^{-5}$	>30000 ^d	$<1.3 \times 10^{-5}$	NA ^e	NA

^aHuman 293HEK embryonic kidney cells were transiently transfected with recombinant human and zebrafish ER DNA constructs together with the luciferase reporter gene PERE-tata-Luc. ^bEC₅₀ is nominal concentration at which 50% of maximal response is reached. ^cEEF is the ratio EC_{50(E2)}/EC_{50(compd)}. ^dEC₅₀ could not be calculated as no or minimal luciferase induction was observed; higher concentrations caused (cyto)toxicity. ^eNA = not analyzed.

dependent manner in the ER-CALUX and showed weak estrogenic activity in the transgenic zebrafish assay (Figure 1; Table 1). However, exposure to nonylphenol (NP) only slightly induced luciferase in transgenic zebrafish (13-fold induction at 1000 nM or 1% of the response of 10 nM E2), whereas it was a full agonist in the ER-CALUX assay (Figure 1). The modest response of the transgenic zebrafish to NP is surprising, as a number of reports have documented the in vivo estrogenicity of nonylphenol in other fish species (reviewed in ref 5). NP has been shown to induce vitellogenesis and inhibition of testicular growth in male rainbow trout exposed under continuous flow conditions for 3 weeks (19) as well as to induce feminized gonads in male Japanese medaka exposed during a full life cycle (3 months) (20). Although it is possible that the 96-h-exposure duration of the transgenic zebrafish was too short to achieve an internal dose that could induce luciferase, prolonged (3 week) exposure to NP also did not result in increased luciferase expression in juvenile transgenic zebrafish (unpublished results). Accordingly, a recent study has shown that despite the high sensitivity of zebrafish to ethynylestradiol, exposure to the alkylphenol 4t-octylphenol failed to induce vitellogenesis in zebrafish (21). These results indicate that zebrafish may show a different sensitivity to alkylphenols than other fish species.

The phthalate DEHP demonstrated very weak estrogenic potency in both the ER-CALUX and the transgenic zebrafish assay (Figure 1). In the ER-CALUX assay, 10000 nM DEHP induced luciferase ~13 times above solvent controls (or ~13% of maximal E2 levels). In the transgenic zebrafish assay, 5000 nM (nominal) DEHP induced luciferase ~20 times above solvent controls (or ~2% luciferase activity relative to 10 nM E2). To our knowledge, specific estrogenic effects of DEHP in fish in vivo have not been demonstrated previously, although the rapid metabolism of DEHP in rainbow trout has been shown (22). In mammals, a recent study has shown the inability of DEHP to induce estrogenic activity (i.e., increase uterine wet weight) in rats in vivo (23). These studies, taken together with our results, suggest that DEHP may not exert estrogenic effects in fish at levels found in the aquatic environment.

Relative Differences in Transactivation Potential of Human and Zebrafish ERs. The large differences in potency

of (xeno)estrogens between the in vitro and in vivo assays may be explained in part by the differential potency of (xeno)-estrogens to activate human and zebrafish ERs. Species-specific differences in response may occur due to differences in transcription factors and the cellular context regulating ER binding and transactivation (24). To investigate these differences, we cloned zebrafish ER α and β subtypes, as well as a zER γ subtype, which was recently demonstrated for the first time in the marine fish Atlantic croaker (25). Zebrafish and human ERs were compared using transient transfection experiments in a human embryonic cell line, which is easily transfected and devoid of steroid hormone receptors and basal metabolic capacity and has been shown previously to be highly responsive to estrogens when recombinant receptor and reporter gene constructs are transfected (11). All test compounds induced dose-response-related luciferase activity in HEK293 cells transiently transfected with ER constructs (Figure 2). When results by E2 exposure were compared, zebrafish ER α was ~40 times less sensitive to E2 than human ER α , whereas zebrafish ER β showed 6 times less sensitivity relative to human ER β (Table 2). Accordingly, rainbow trout ER has been shown to require 10 times higher E2 concentrations than the human ER for transactivation (26, 27). Although the zebrafish ERs demonstrated a lower transactivational potential following estrogen exposure as compared to human ERs, an interesting difference was observed in the relative potency for E2 between human and fish ER β subtypes. E2 transactivated zebrafish ER α and ER β at similar concentrations, whereas a ~10-fold excess of E2 was required to transactivate human ER β both in this study (Table 2) and in others (28). These results suggest that the ER β subtype in zebrafish may be relatively more sensitive to (xeno)estrogens than the human ER β . In addition, zebrafish ER γ was activated by the lowest E2 concentrations of the three zebrafish receptor subtypes (EC₅₀ = 0.13 nM; Table 2), suggesting the important role of this receptor in estrogen regulation in fish. Estrone transactivated both human and zebrafish ER α and ER β at similar potency relative to E2 (Table 2). The high activity of EE2 shown in the in vivo transgenic zebrafish assay was also observed in the in vitro transfection assays in which a relatively high transactivation potential of EE2 was found; EE2 was up to 4 times more potent than E2 in transactivating zebrafish ER α , β , and γ subtypes (Table 2). Accordingly, in

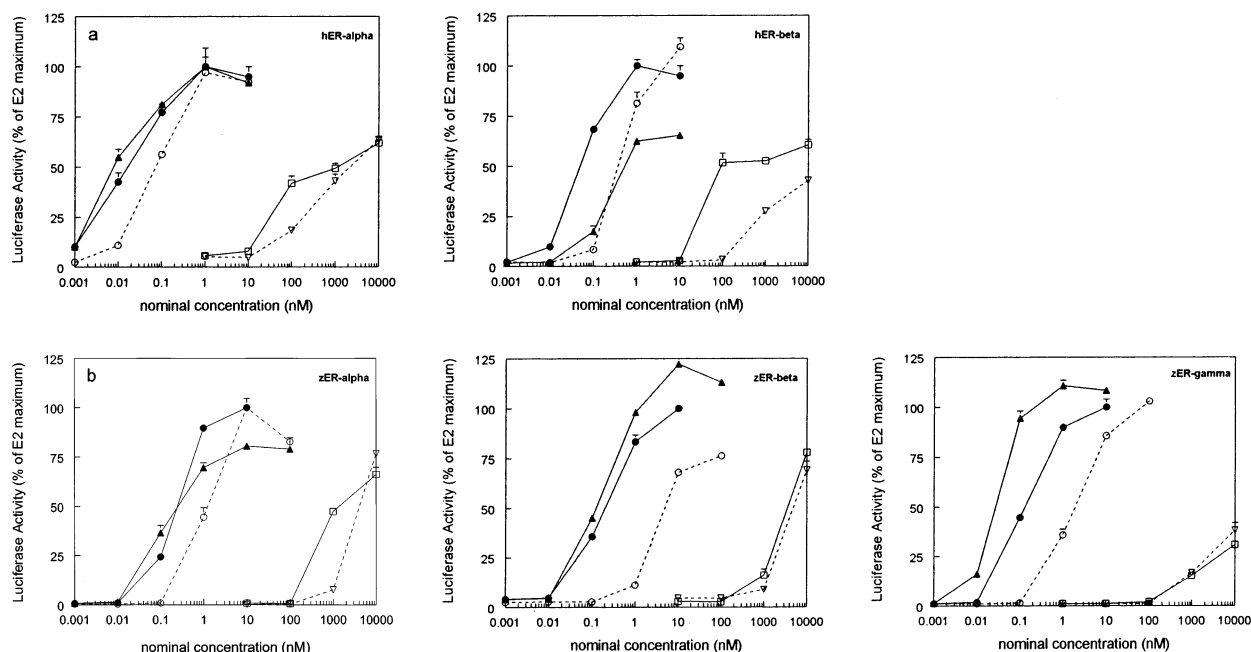


FIGURE 2. Transactivation of (A) human estrogen receptor α and β and (B) zebrafish estrogen receptors α , β , and γ by (xeno)estrogens estradiol (E2, ●), estrone (E1, ○), ethynylestradiol (EE2, ▲), *o,p'*-DDT (▽), and 4-nonylphenol (NP, □). Human HEK293 cells were transiently transfected with human or zebrafish ER and luciferase reporter gene constructs. Data are the average of two to three independent assays. Values given are mean; error bars represent standard error of the mean ($n = 3$).

binding studies with channel catfish ER, EE2 was found to be 5 times more active than E2 (29). The xenoestrogens tested showed similar weak agonistic (*o,p'*-DDT, NP) or lack of (DEHP) estrogenic activity in ERs from both species. Interestingly, NP did induce dose-response-related transactivation of all three zebrafish ERs (Figure 2b), indicating that the lack of response of the transgenic zebrafish to NP may be due to other factors (see below).

Differences in Target Cell Exposure in the Screening Assays. In addition to differences at the receptor level, differences in reporter gene activity following exposure to (xeno)estrogens between the in vitro ER-CALUX screening assay and the in vivo transgenic zebrafish assay may also be explained by the lower actual target cell exposure in the transgenic fish. Target cell concentrations depend on the biotransformation and toxicokinetics of the compound in the fish as well as its fate in the aquarium. In vivo, natural estrogens as well as NP can be extensively transformed to less potent metabolites by fish (30, 31). Therefore, in the transgenic zebrafish, higher concentrations of E2, E1, or NP may be required to induce luciferase in target cells. In the ER-CALUX assay, biotransformation during the 24-h exposure period is likely to be much lower. The high potency of ethynylestradiol in vivo in the transgenic zebrafish may be partly due to its higher persistence in vivo compared to E2 and E1, as it is poorly metabolized by the liver and subject to intensive enterohepatic recycling (32). The lipophilic compound *o,p'*-DDT was found in extremely high levels in fish tissues following the 96-h exposure period ($>600 \mu\text{g/kg}$; Table 3), indicating that bioaccumulation potential is also an important factor in predicting differences between in vitro and in vivo responses.

In addition to differences in biotransformation and toxicokinetics of the test compounds, the strategy for dosing chemicals differs greatly between the two assays. In the ER-CALUX assay, chemicals are added to sterile medium containing serum with lipids and proteins, which will mediate the cellular availability and membrane transport of test chemicals. In the transgenic zebrafish assay, chemicals are added directly to water and may rapidly disappear from the

TABLE 3. Concentrations of (Xeno)estrogens Determined in Test Water and Fish Taken during Exposure Experiments with Transgenic Zebrafish^a

compd	nominal concn,		measured concn			
	water		water		transgenic zebrafish	
	nM	$\mu\text{g/L}$	$t = 0$	$t = 96 \text{ h}$	$t = 0$	$t = 96 \text{ h}$
E2	100	27	20.3	1	NA ^b	
EE2	1	0.3	0.4	<dl ^c	NA	
E1	100	27	30	0.2	NA	
NP	1000	220	133	7	<dl	8940
<i>o,p'</i> -DDT	1000	346	340	850	<dl	685000

^aSamples were taken at the start ($t = 0$) and end ($t = 96 \text{ h}$) of the experiment. ^bNA = not analyzed. ^c<dl = below detection limit.

water column due to sorption on glass, fish, and detritus as well as biodegradation, resulting in large deviations between nominal and actual test concentrations. Indeed, with the exception of *o,p'*-DDT, chemical analysis of actual test compound levels in exposure water revealed that although actual concentrations were similar to nominal concentrations at the beginning of the experiment, very low amounts of the (xeno)estrogens were present after 96 h of exposure, despite daily renewal of the exposure water (Table 3). In the case of *o,p'*-DDT, actual concentrations were higher at the end of the 96-h exposure period, which can be explained by the liquid-liquid extraction procedure used for DDT analysis in contrast to the solid phase extraction procedure used for the other test chemicals. DDT absorbed to particles in the water column will also be extracted, leading to an overestimation of the amount of dissolved *o,p'*-DDT. Despite the difficulty in maintaining constant aqueous concentrations of test chemicals, a static renewal experimental design has been widely used for toxicity testing and has definite advantages because it allows rapid, inexpensive, and easy screening of chemicals in virtually any laboratory situation. However, an experimental design using a flow-through system with continuous dosing of chemicals will likely result in higher

sensitivity to test compounds, as well as actual test concentrations which more realistically reflect nominal concentrations. To this end, more frequent measurements of actual test concentrations during the exposure period are also recommended.

Use of in Vitro and in Vivo Reporter Gene Assays To Screen Estrogenic Activity. Both the in vitro ER-CALUX assay and the in vivo transgenic zebrafish assay have a number of characteristics that make them useful screening models. The ER-CALUX assay is extremely sensitive and rapid and can be used to screen many chemicals in a high-throughput manner. The ER-CALUX assay is a suitable choice for screening new and existing chemicals, as well as complex environmental samples for unexpected estrogenic activity that may be further identified by chemical analysis. The high sensitivity of the ER-CALUX assay minimizes the potential for false-negative results. However, false-positives may occur primarily due to the fact that an in vitro assay may poorly predict the toxicokinetics of a substance in vivo, as was shown in this study for EE2 and *o,p'*-DDT. In addition, differential receptor activation and ligand specificity was observed between human and zebrafish ERs, emphasizing the need for heterologous systems for risk assessment purposes. For these reasons, the in vivo transgenic zebrafish assay can form an excellent complement to the ER-CALUX assay. The assay is rapid (96 h) and easy to perform, and the measured endpoint (luciferase activity) is simple and cost-effective. The value of the transgenic zebrafish assay is that it can predict if estrogenic activity measured with an in vitro assay actually may affect fish during a critical life stage, for example, the stage of gonad differentiation. When adult transgenic fish are used, it is possible to determine the tissue-specific effects of (xeno)estrogens (9). The transgenic zebrafish assay may be used to further direct and fine-tune costly long-term exposure studies in which reproductive and histological parameters are analyzed. Ideally, biological validation of the ER-CALUX and transgenic zebrafish assays will reveal critical levels of reporter gene induction that correspond with estrogenic effects on gonad differentiation and reproduction in the zebrafish.

Acknowledgments

We acknowledge the contribution of Dr. Wang Deshou, National Institute for Basic Biology, Japan, in the cloning of the zebrafish estrogen receptors. We thank N. Jonkers, Department of Environmental Toxicology and Chemistry, University of Amsterdam, for his assistance with the nonylphenol analysis. Analysis of *o,p'*-DDT was carried out in the group of Dr. P. Leonards at Netherlands Institute for Fisheries Research, IJmuiden. The Dutch Ministry of Transport, Public Works and Water Management (RWS/RIKZ) and the Dutch Technology Foundation (STW) financially supported this study.

Literature Cited

- (1) Lye, C. M.; Frid, C. L. J.; Gill, M. E.; McCormick, D. *Mar. Pollut. Bull.* **1997**, *34*, 34–41.
- (2) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. *Environ. Sci. Technol.* **1998**, *32*, 2498–2506.
- (3) Allen, Y.; Scott, A. P.; Matthiessen, P.; Haworth, S.; Thain, J. E.; Feist, S. *Environ. Toxicol. Chem.* **1999**, *18*, 1791–1800.
- (4) Vethaak, A. D.; Rijs, G. B. J.; Schrap, S. M.; Ruiter, H.; Gerritsen, A.; Lahr, J. *Estrogens and Xeno-estrogens in the Aquatic Environment of The Netherlands: Occurrence, Potency and Biological Effects*; RIKZ Report 2002.001; Ministry of Transport and Public Works: The Hague, The Netherlands, 2002; in press.
- (5) Tyler, C. R.; Jobling, S.; Sumpter, J. P. *Crit. Rev. Toxicol.* **1998**, *28*, 319–361.
- (6) Zacharewski, T. R. *Environ. Sci. Technol.* **1997**, *31*, 613–623.
- (7) Ankley, G. T.; Mihaich, E.; Stahl, R.; Tillitt, D.; Colborn, T.; McMaster, S.; Miller, R.; Bantle, J.; Campbell, P.; Denslow, N.; Dickerson, R.; Folmar, L.; Fry, M.; Giesy, J.; Gray, L. E.; Guiney, P.; Hutchinson, T.; Kennedy, S.; Kramer, V.; LeBlanc, G.; Mayes, M.; Nimrod, A.; Patino, R.; Peterson, R.; Purdy, R.; Ringer, R.; Thomas, P.; Touart, L.; Van der Kraak, G.; Zacharewski, T. R. *Environ. Toxicol. Chem.* **1998**, *17*, 68–87.
- (8) Legler, J.; Van den Brink, C. E.; Brouwer, A.; Murk, A. J.; Van der Saag, P. T.; Vethaak, A. D.; Van der Burg, B. *Toxicol. Sci.* **1999**, *48*, 55–66.
- (9) Legler, J.; Broekhof, J.; Brouwer, A.; Lanser, P.; Murk, A. J.; Van der Saag, P.; Vethaak, A. D.; Wester, P.; Zivkovic, D.; Van der Burg, B. *Environ. Sci. Technol.* **2000**, *34*, 4439–4444.
- (10) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
- (11) Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B.; Gustafsson, J. A. *Endocrinology* **1998**, *139*, 4252–4263.
- (12) Belfroid, A. D.; Van der Horst, A.; Vethaak, A. D.; Schafer, A.; Rijs, G. B. J.; Wegener, J.; Cofino, W. P. *Sci. Total Environ.* **1999**, *225*, 101–108.
- (13) De Voogt, P.; de Beer, K.; Van der Wielen, F. W. M. *Trends Anal. Chem.* **1997**, *16*, 584–595.
- (14) Zhao, M.; Van der Wielen, F. W. M.; De Voogt, P. *J. Chromatogr. A* **1999**, *837*, 129–138.
- (15) Panter, G. H.; Thompson, R. S.; Sumpter, J. P. *Aquat. Toxicol.* **1998**, *41*, 243–253.
- (16) Routledge, E. J.; Sheahan, D.; Desbrow, C.; Brighty, G. C.; Waldock, M.; Sumpter, J. P. *Environ. Sci. Technol.* **1998**, *32*, 1559–1565.
- (17) Metcalfe, C. D.; Metcalfe, T. L.; Kiparissis, Y.; Koenig, B. G.; Khan, C.; Hughes, R. J.; Croley, T. R.; March, R. E.; Potter, T. *Environ. Toxicol. Chem.* **2001**, *20*, 297–308.
- (18) Lange, R.; Hutchinson, T. H.; Croudace, C. P.; Siegmund, F.; Schweinfurth, H.; Hampe, P.; Panter, G.; Sumpter, J. P. *Environ. Toxicol. Chem.* **2001**, *20*, 1216–1227.
- (19) Jobling, S.; Sheahan, D.; Osborne, J. A.; Matthiessen, P.; Sumpter, J. P. *Environ. Toxicol. Chem.* **1996**, *15*, 194–202.
- (20) Gray, M. A.; Metcalfe, C. D. *Environ. Toxicol. Chem.* **1997**, *16*, 1082–1086.
- (21) Van den Belt, K.; Verheyen, R.; Witters, H. *Arch. Environ. Contam. Toxicol.* **2001**, *41*, 458–467.
- (22) Barron, M. G.; Tarr, B. D.; Hayton, W. L. *Toxicol. Appl. Pharmacol.* **1987**, *88*, 305–312.
- (23) Zacharewski, T. R.; Meek, M. D.; Clemons, J. H.; Wu, Z. F.; Fielden, M. R.; Matthews, J. B. *Toxicol. Sci.* **1998**, *46*, 282–293.
- (24) Petit, F.; Le Goff, P.; Cravedi, J.-P.; Valotaire, Y.; Pakdel, F. *J. Mol. Endocrinol.* **1997**, *19*, 321–335.
- (25) Loomis, A. K.; Thomas, P. *Biol. Reprod.* **1999**, *61*, 51–60.
- (26) Petit, F.; Valotaire, Y.; Pakdel, F. *Eur. J. Biochem.* **1995**, *233*, 584–592.
- (27) Le Drean, Y.; Kern, L.; Pakdel, F.; Valotaire, Y. *Mol. Cell. Endocrinol.* **1995**, *109*, 27–35.
- (28) Lemmen, J. G.; Van den Brink, C. E.; Legler, J.; Van der Saag, P. T.; Van der Burg, B. *J. Endocrinology* **2002**, *174*, 435–446.
- (29) Nimrod, A. C.; Benson, W. H. *Crit. Rev. Toxicol.* **1996**, *26*, 335–364.
- (30) Bone, Q.; Marshall, N. B.; Blaxter, J. H. S. *Biology of Fishes*; Blackie Academic and Professional: London, U.K., 1995; pp 194–218.
- (31) Lewis, S. K.; Lech, J. J. *Xenobiotica* **1996**, *26*, 813–819.
- (32) Guengerich, F. P. *Life Sci.* **1990**, *47*, 1981–1988.

Received for review December 17, 2001. Revised manuscript received July 16, 2002. Accepted July 29, 2002.

ES010323A